CHEMISTRY OF NATURAL PRODUCTS

DISSERTATION

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BY

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This is to certify that the work described in the dissertation entitled CHEMISTRY OF NATURAL PRODUCTS is the original work of the candidate and is suitable for submission for the award of M. Phil. degree in Chemistry.

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INTRODUCTION

In common with the studies of naturally occurring compounds of all kinds, flavonoid chemistry has emerged from undirected search for new compounds and the establishment of their structures, by conventional means. The term flavonoid covers a large group of naturally occurring compounds in which two benzene rings are linked by a propane bridge (C_6-C-C-C-C_6) except in isoflavones in which the arrangement is (C_6-C-C(-C_6)-C). The flavonoids include chalcones, dihydrochalcones, aurones, flavanones, flavones, isoflavones, flavonols, 2,3-dihydroflavonols (flavononols), flavan-3,4-diols (leucoanthocyanidins), anthocyanidins, catechins, and tetraflavonoids[1].

Numerous physiological activities have been attributed to flavonoids[2]. Some biological and pharmacological actions of flavonoids have been described in a recent article[3] like diuretic action, treatment of allergy, protection against X-rays and other radiation injuries, cure of frostbite, antibacterial activity, prophylactic action, oestrogenic activity, antitumor effects and antioxidant property. Robinetin and Gossypetin were claimed as the most potent and of economic importance in the tanning of leather, the fermentation of tea,

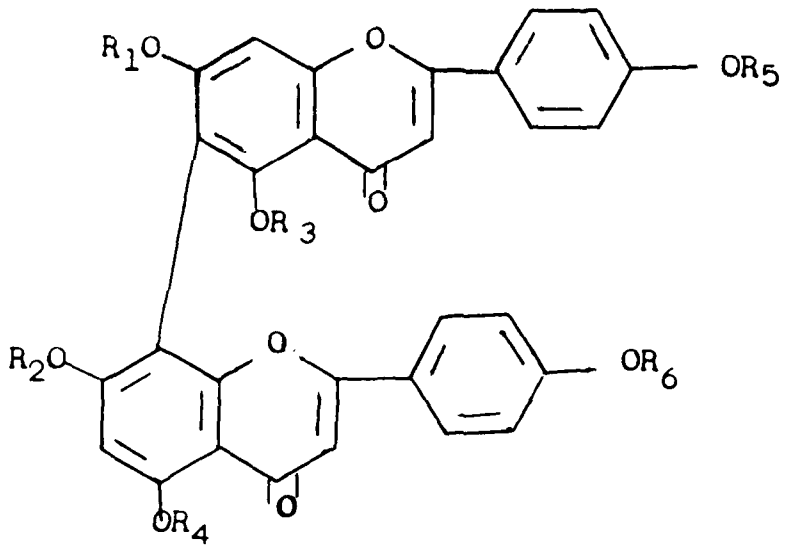
the manufacture of cocoa and in the flavour qualities of food stuffs[4]. Some flavonoids, such as eupatin, eupifolin are active against Eagles carcinoma of nasopharynx[4A] carried out in the cell cultures.

Biflavonoids are the dimer of flavonoids and derived from two flavone or flavanone or flavanone-flavone units and have been mostly isolated from Gymnosperms. Among the angiosperms, some plants belonging to clausiaceae (Guttifereae)[5,6] Euphorbiaceae[7-9], Caprifoliaceae[10], Archegoniaceae[11], Ochnaceae[12], Anacardiaceae[13] and some ferns belonging to Selaginillaceae[14] have been found to contain biflavonoids.

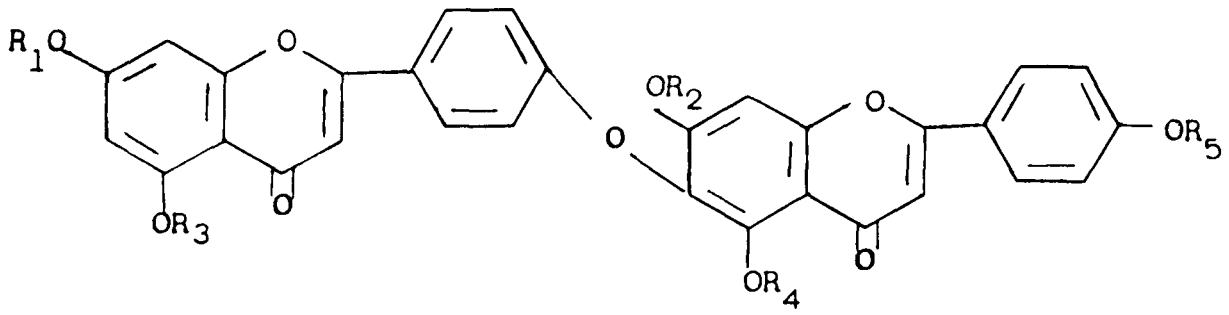
CLASSIFICATION OF BIFLAVONOIDS

The naturally occurring biflavonoids may be classified into two groups :

[A] The Biphenyl Type :



[B] The Biphenyl Ether Type :



[A] Biphenyl Type Biflavonoids :

(1) Agathisflavone series

These are derived from two apigenin units with (I-6, II-8) linkage, and are represented by five members with agathisflavone as a parent compound.

		R_1	R_2	R_3	R_4	R_5	R_6
(a)	Agathisflavone[15,16]	H	H	H	H	H	H
(b)	I-7-O-methyl[17-19]	CH_3	H	H	H	H	H
(c)	I-7,II-7-di-O-methyl[20]	CH_3	CH_3	H	H	H	H
(d)	II-4',I-7-di-O-methyl[19,17]	CH_3	H	H	H	H	CH_3
(e)	II-4',I-7,II-7-tri-O-methyl[21]	CH_3	CH_3	H	H	H	CH_3

(2) Amentoflavone series

These are derived from two apigenin units with (I-3',II-8) linkage and are represented by sixteen members with amentoflavone as the parent compound.

	R_1	R_2	R_3	R_4	R_5	R_6
(a) Amentoflavone[22-24]	H	H	H	H	H	H
(b) I-7-O-methyl[25-27] (Sequoiaflavone)	CH_3	H	H	H	H	H
(c) I-4'-O-methyl[27-29] (Bilobetin)	H	H	H	H	CH_3	H
(d) II-7-O-methyl[30,15]	H	CH_3	H	H	H	H
(e) II-4'-O-methyl[31] (Podocarpusflavone-A)	H	H	H	H	H	CH_3
(f) I-4',I-7-di-O-methyl[26,27,22] (Ginkgetin)	CH_3	H	H	H	CH_3	H
(g) I-4',II-4'-di-O-methyl[26,28,31] (Isoginkgetin)	H	H	H	H	CH_3	CH_3
(h) II-4',I-7-di-O-methyl[31] (Podocarpusflavone-B)	CH_3	H	H	H	H	CH_3
(i) I-4',II-7-di-O-methyl[33]	H	CH_3	H	H	CH_3	H
(j) I-7,II-7-di-O-methyl[34a,b]	CH_3	CH_3	H	H	H	H
(k) II-4',I-7,II-7-tri-O-methyl[18,35] (Heveaflavone)	CH_3	CH_3	H	H	H	CH_3
(l) I-4',II-4',II-7-tri-O-methyl[33-36] (Kayaflavone)	H	CH_3	H	H	CH_3	CH_3

(m) I-7, I-4', II-4'-tri-O-methyl[26-28,32] (Sciadopitysin)	CH_3	H	H	H	CH_3	CH_3
(n) I-4', I-7, II-7-tri-O-methyl[34]	CH_3	CH_3	H	H	CH_3	H
(o) I-4', II-4', I-7, II-7-tetra-O-methyl[37-39]	CH_3	CH_3	H	H	CH_3	CH_3
(p) I-4', II-4', I-5, II-5, I-7, II-7-hexa-O-methyl[40]	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3

(3) I-2,3-Dihydro-amentoflavone series[41-42]

These are derived from naringenin and apigenin units with (I-3', II-8) linkage and are represented by three members with dihydroamentoflavone as the parent compound and its two partial methyl ethers.

	R_1	R_2	R_3	R_4	R_5	R_6
(a) I-2,3-di-hydroamento-flavone[41-43]	H	H	H	H	H	H
(b) II-4', II-7-di-O-methyl[41-43]	H	CH_3	H	H	H	CH_3
(c) I-4', II-4, I-7-tri-O-methyl[43]	CH_3	H	H	H	CH_3	CH_3

(4) Tetrahydro-amentoflavone series[44]

Three new closely related biflavones A B and C have been isolated from defatted nuts of Semicarpus anacardium[44] . The first of these has been characterised as its methyl ethers A_1 and A_2.

		R
(A_1)	I-7,I-4',II-4'-tri-O-methyl I-5,II-5,II-3'-trihydroxy(I-3',II-8) biflavanone	H
(A_2)	I-7,I-4',II-4',II-3'-tetra-O-methyl I-5,II-5-dihydroxy(I-3',II-8) biflavanone	Me

The biflavanones B and C have also been characterised as their methyl ethers/corresponding chalcone methyl ethers.

Suggested structures are O-methyl derivatives of (I-3',II-8)binaringenin and (I-3',II-8)biliquiritigenin respectively.

(5) I-7-O-methyl, I-6-C-methyl amentoflavone[45]

Rahman et al. have isolated 7-O-methyl, I-6-C-methyl amentoflavone from the leaves of Cephalotaxus haringtonia K. Koch. This is derived from 6-C-methylgenkwanin and apigenin with (I-3',II-8)linkage.

(6) Cupressuflavone series[46]

These are derived from two apigenin units with (I-8,II-8) linkage and are represented by seven members. Cupressuflavone[46] is the parent compound while others are its

partial methyl ethers.

		R_1	R_2	R_3	R_4	R_5	R_6
(a)	Cupressuflavone[46]	H	H	H	H	H	H
(b)	I-7-O-methyl[20,16]	CH_3	H	H	H	H	H
(c)	I-7,II-7-di-O-methyl[20,16]	CH_3	CH_3	H	H	H	H
(d)	I-4',I-7 (or II-4',I-7) di-O-methyl[34]	CH_3	H	H	H	H/CH_3	CH_3 H
(e)	I-4',I-7,II-7-tri-O-methyl[33]	CH_3	CH_3	H	H	CH_3	H
(f)	I-4',II-4',I-7,II-7-tetra-O-methyl[47]	CH_3	CH_3	H	H	CH_3	CH_3
(g)	I-4',II-4',I-5,I-7,II-7-Penta-O-methyl[48] (Synthetic compound)						

The structure of I-4'-di-O-methyl cupressuflavone, isolated from Araucaria cunninghamii and A. cookii[38], has been revised to I-7,II-7-di-O-methyl cupressuflavone[38].

(7) Robustaflavone[49] series :

These are derived from two apigenin units with (I-3,II-6) linkage and are represented by three members. Robustaflavone is the parent compound and rest two are its mono- and di- methyl ethers[49].

(8) BGH series[50]

These are derived from naringenin and apigenin or luteolin unit with (I-3,II-8) linkage and are represented by BGH-II and BGH-III as the parent compounds respectively.

		R_1	R_2	R_3	R_4	R_5	R_6
(a)	BGH-II (Morelloflavone)[5,50,51]	OH	H	H	H	H	H
(b)	II-3'-O-methyl-fukugetin[57]	OCH_3	H	H	H	H	H
(c)	BGH-III (Talbotaflavone/ volkensiflavone)[5,54-56]	H	H	H	H	H	H

I-4',II-4',I-7,II-7-tetra-O-methyl-, II-3'-methoxy[52] and II-4',I-7,II-7-tri-O-methyl, II-3'-methoxy[53] are synthetic compounds of above series.

(9) GB series[6,57-60]

This series comprises of reduced heterocyclic systems. The following members are reported, to occur in nature. They are derived from naringenin linked with a naringenin or aromodendrin or taxifolin or eriodictyol through (I-3,II-3) linkage.

(a) GB-1 $R_1 = R_2 = H, \quad R_3 = OH$

(b) GB-1a $R_1 = R_2 = R_3 = H$

(c) GB-2 $R_1 = R_2 = OH, \quad R_3 = H$

(d) GB-2a $R_1 = R_3 = H, \quad R_2 = OH$

(e) II-4'-O-methyl-GB (Koloflavanone) $R_2 = R_3 = OH$, $R^e = Me$

(f) Manniflavanone $R_1 = R_2 = R_3 = OH$

(g) GB-3 $R_1 = R_2 = R_3 = OH$, $R^b = Me$

(10) I-4',II-4',I-5,II-5,I-7,II-7-Hexahydroxy(I-3,II-3) biflavone[61] :

This series comprises of only one member and has been synthesised by oxidative coupling of apigenin[54].

(11) Mesuaferrone - A[62] :

Mesuaferrone - A has been isolated from the stamens of Mesua ferrea Linn. This is derived from two naringenin units with (I-8,II-8) linkage.

(12) Mesuaferrone - B[62] :

This is derived from a naringenin and apigenin unit through (I-8,II-8) linkage.

(13) Rhusflavanone[63] :

Rhusflavanone has been isolated from the seed kernel of Rhus succedanea. This is derived from two naringenin units with (I-6,II-8) linkage.

(14) Rhusflavone[64] :

This is derived from naringenin and apigenin units linked through (I-6,II-8).

(15) Succedanea flavanone[65] :

This is derived from two naringenin units with (I-6,II-6) linkage.

(16) I-3,II-3 flavanone series[66] :

The series consists of two C-C linked naringenin (5,7,4-trihydroxyflavanone) units. Usually these possesses I-3,II-3 interflavonoid linkage which may either be α or β-oriented and comprises of almost ten members.

(a)	Chamae-jasmine	$R_1 = R_2 = \alpha$-H, $R_3 = \beta$-H
(b)	Chamae-jasmine A	$R_1 = R_4 = H$, $R_2 = \alpha$-H, $R_3 = \beta$-H
(c)	Chamae-jasmine B	$R_1 = R_2 = \beta$-H, $R_3 = H$, $R_4 = Me$
(d)	Chamae-jasmine C	$R_1 = R_2 = \beta$-H, $R_3 = R_4 = Me$
(e)	Isochamae-jasmine	$R_1 = R_3 = \alpha$-H, $R_2 = \beta$-H
(f)	Neo-chamae-jasmine A	$R_1 = R_2 = R_3 = \beta$-H
(g)	Neo-chamae-jasmine B	$R_1 = R_2 = \beta$-H, $R_3 = \alpha$-H
(h)	Sikokiamin A	$R_1 = R_2 = \beta$-H, $R_3 = R_4 = H$
(i)	Sikokiamin B	$R_1 = \beta$-H, $R_2 = \alpha$-H, $R_3 = R_4 = H$
(j)		$R_1 = R_4 = Me$, $R_2 = \beta$-H, $R_3 = \alpha$-H

(17) I-4',II-4',I-5,II-5,II-7-Pentahydroxy, I-7-O-methyl, I-6-C-methyl (I-3',II-8)biflavone[67] :

This is derived from 6-C-methyl genkwanin and apigenin with (I-3',II-8) linkage.

(18) I-4',I-5,II-5,I-7,II-7-Pentahydroxyflavanone (I-3,II-8)chromone[57] :

The compound has been isolated from leaves of Garcinia dulcis (Kurz.). It is a dimer of naringenin and 5,6-dihydroxychromone linked through (I-3,II-8). Its isolation has introduced a new series comprising of flavanone-chromone structure.

(19) Taiwaniaflavone series :

A new series of naturally occurring biflavones have been isolated from Taiwania cryptomerioides[63] Hataya. Taiwaniaflavone as the parent and its mono and dimethyl ethers. These are derived from two apigenin units with (I-3',II-3) linkage.

		R_1	R_2	R_3	R_4	R_5	R_6
(a)	Taiwaniaflavone[63]	H	H	H	H	H	H
(b)	I-7/II-7-O-methyl[63]	H/CH_3	CH_3/H	H	H	H	H
(c)	II-4',I-7/II-7-di-O-methyl[63]	H/CH_3	CH_3/H	H	H	H	CH_3

(d) I-4',II-4',I-5,II-5,I-7,II-7-Hexa-O-methyl-taiwaniaflavone (a synthetic compound).

(20) Isoflavonoflavones[68] :

Two representatives namely Bryoflavanone and Heterobryoflavanone having C-C bonded isoflavone (orobol-5, 7,3',4'-tetrahydroxyisoflavone) and flavone (luteolin-5,7, 3',4'-tetrahydroxyisoflavone) have been recently isolated from the Moss Bryum capillare and represent I-3', II-6 and I-3', II-8 types.

(21) Isobiflavonoids[69] :

Lophinone A is the only example of this sub-category which differs from other biflavonoids.[69]

[B] Biphenyl Ether Type Biflavonoids :

(1) Hinokiflavone series :

These are derived from two apigenin units with (I-4'-O-II-6) linkage. Hinokiflavone is the parent compound with six others as its partial methyl ethers. Earlier hinokiflavone and its derivatives were assigned (I-4'-O-II-8)[58] linkage which had later been revised to (I-4'-O-II-6)[59,61,70,71].

		R_1	R_2	R_3	R_4	R_5	R_6
(a)	Hinokiflavone[58-59,71]	H	H	H	H	H	H
(b)	I-7-O-methyl[31] (Neocryptomerin)	CH_3	H	H	H	H	H
(c)	II-7-O-methyl[61] (Isocryptomerin)	H	CH_3	H	H	H	H
(d)	II-4'-O-methyl[70] (Cryptomerin)	H	H	H	H	H	CH_3
(e)	I-7,II-4'-di-O-methyl[70]	CH_3	H	H	H	H	CH_3
(f)	I-7,II-7-di-O-methyl[31]	CH_3	CH_3	H	H	H	H

II-4',II-7, di-O-methyl (cryptomerin-B)[70] and I-7,II-7, II-4'-tri-O-methyl[70] are synthetic compounds of above series.

(2) I-2,3-Dihydrohinokiflavone series :

The sole member of this series has been isolated from Metasequioia glyptostroboidies and Cycas species[43, 72].

(3) Ochnaflavone series[73, 12] :

Ochnaflavone is the parent compound with three others as its partial methyl ethers. They are derived from two apigenin units with (I-3'-O-II-4') linkage.

		R_1	R_2	R_3	R_4	R_5
(a)	Ochnaflavone[12]	H	H	H	H	H
(b)	I-4'-O-methyl ether[12]	H	H	H	H	CH_3
(c)	I-4',I-7-di-O-methyl ether[12,73]	CH_3	H	H	H	CH_3
(d)	I-4',I-7,II-7-tri-O-methyl ether[12,73] (Synthetic)	CH_3	CH_3	H	H	CH_3

(4) Podoverine C[74] :

Poroverine C represents the subcategory of biflavonoids having C–O–C linkage between C-4' and C-3 of a 3-methoxy flavone and 2,3-dihydroxyflavanone moieties, has been recently characterised from Podophyllum versipelle cell culture.

THEORETICAL

STRUCTURE DETERMINATION OF BIFLAVONOIDS :

The problem of structure determination of biflavonoids is a complex one because of (a) occurrence of more than one biflavonoid in chromatographically homogeneous fractions with the consequent difficulty in their isolation in pure form, (b) insolubility in usual organic solvents, (c) the difficulty in exact location of O-methyl in partially methylated derivatives of biflavones and (d) the intricate problem of establishing the inter-flavonoid linkage.

There are various methods generally used for structure determination such as colour reaction, degradation[20,30,34], physical methods and synthesis. The physical methods and synthesis are of key importance for complete structure elucidation of biflavonoids.

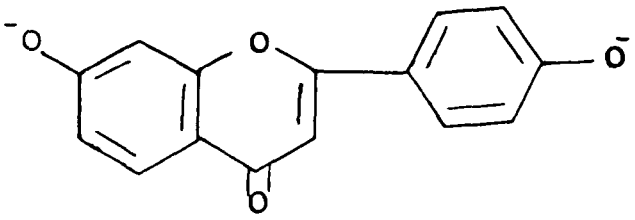
PHYSICAL METHODS :

The physical methods generally employed in the identification and structural analysis of plant pigments are chromatography[75-80], UV[81], IR[82-85], ^{1}H-NMR[102-105], ^{13}C-NMR[101-108] and mass spectrometry[109-112]. Among them the

nuclear magnetic re-sonance spectroscopy and mass spectrometry are most sophisticated dependable tools for the structure determination of flavonoids.

(a) INFRARED SPECTROSCOPY[86-89] :

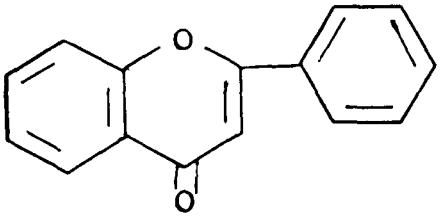
The infrared spectra of 5-hydroxyflavones show strong bands at 1660 cm^{-1} as do those of mono-5-hydroxyflavone and although this hydroxyl group is internally hydrogen-bonded the effect of 5-O-alkylation and 5-O-acylation is opposite to that shown in the case of simple O-hydroxy ketones, because of internal hydrogen bonding in O-hydroxyl ketones. The carbonyl bands of these compounds show a shift to higher frequencies on either O-alkylation or O-acylation. However, a similar comparison of the infrared spectra of 5-hydroxyflavones and 5-hydroxychromones with the spectra of their 5-O-alkyl, and 5-O-acyl derivatives shows a shift in the opposite direction, that is to lower frequencies. In practice this effect is very useful in diagnosing the presence of a 5-hydroxyl flavone structure.



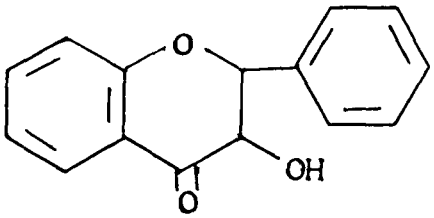
(b) ULTRAVIOLET ABSORPTION SPECTRA :

The U.V. spectra of flavonoids have been thoroughly studied and reviewed by Jurd[90].

Flavones (I) and flavonols (II) generally exhibit high intensity absorption in the 300-380 nm region (band-I) and the 240-270 nm region (band-II)[91-92]. The position and intensity of the max of the absorption bands varies with the relative resonance contributions of the benzoyl (III) cinnamoyl (IV) and pyrone ring (V) groupings to the total resonance of the flavone molecule.



I



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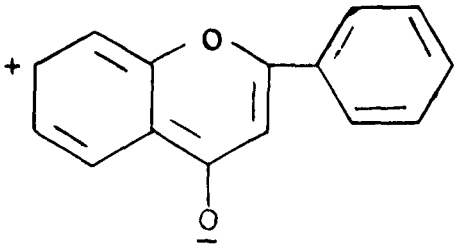

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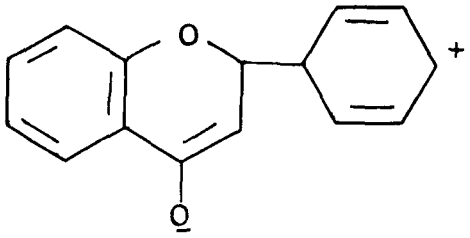

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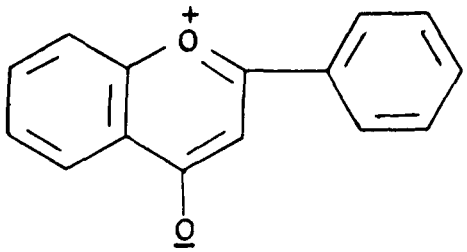


V

Although these grouping interact, the spectra of substituted flavones and flavonols in the neutral and alkaline solutions suggest that band I is associated chiefly with the absorption in the cinnamoyl grouping IV and band II with absorption in the benzoyl grouping[93] (V). The location of the hydroxyl groups are fixed on the basis of their shifts in different shift reagents[94-95].

U.V. SPECTRA OF BIFLAVONOIDS :

Ultraviolet spectroscopy has been found extremely helpful in structure elucidation of biflavonoids.

The UV spectra of biapigenin or binaringenin type biflavonoids and their derivatives are very similar to those constituent monomer unit, with the only difference that molecular extinction co-efficient (Σ) of biflavonoids are approximately double as compared to the corresponding monomers e.q. for amentoflavone (Ginkgetin) $= 4.2 \times 10^{-3}$

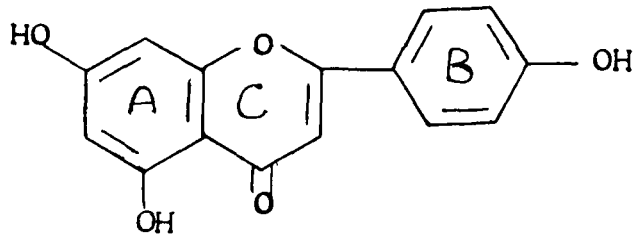
for apigenin $= 2.0 \times 10^{-3}$

(c) NUCLEAR MAGNETIC RESONANCE ^{1}H-NMR SPECTROSCOPY :

The application of NMR spectroscopy has proved to be the most powerful tool in the structure determination of flavonoids. By the use of NMR studies of the silyl derivatives[96], double irradiation technique[46a], solvent induced shift studies[46b, 97, 98], lanthanide induced shift studies[99], nuclear overhauser effect[100] and ^{13}C-NMR spectroscopy[101], one can come to the structure of flavonoid occuring even in minor quantities without tedious and the time consuming chemical degradation and synthesis.

Batterham and Highest[102], Mabry[103], Massicot[104], Clark-lewis[105], Kawano[30,99] and Petter and Rahman[36,37,71] have made their valuable contributions in this field.

The most commonly occurring hydroxylation pattern in natural flavonoid is 4',5,7-trihydroxy system (I). The chemical shifts of the protons of ring A and B prove to be independent of each other but are affected by the nature of ring 'C'.



I

RING - A :

The two A-ring protons of flavonoids with the 5,7-hydroxylation pattern give rise to two doublets (J = 2.5Hz) between τ 3.3-4.0 from tetramethyl silane. There are, however, small but predictable variations in the chemical shifts of the C-6 and C-8 proton signals depending on the 5- and 7-substituents. In flavanones the 6,8 proton give a signal peak near τ 4.05, with the addition of a 3-hydroxy group (flavanonols) the chemical shifts of these protons are slightly altered and the pattern changes to a very strongly coupled pair of doublets. The presence of double bond in ring C of flavones and flavonols causes a marked downfield shift of these peaks, again producing the two doublet pattern out of 6- and 8-protons, the later appears downfield.

RING - B :

All B-ring protons appear around τ 2.3-3.3 a region separate from the usual A-ring protons. The signals from the aromatic protons of an unsubstituted B-ring in a flavanone appear as a broad peak centred at about τ 2.55. In flavones, the presence of 'C' ring double bond causes a shift of the 2',6'-protons and the spectrum shows two broad peaks, one centred at τ 2.00 (2',6') and the other at τ2.4 (3',4',5')[92].

With the introduction of a 4'-hydroxy group, the B-ring protons appear affectively as a four peak pattern, this is called A_2B_2 pattern. Introduction of one more substituent to ring B gives the normal ABC pattern. The hydroxyl group increases the shielding on the adjacent 3',5' protons and their peaks move substantially upfield. The 2',6' protons of flavanones give signals centred at about τ 2.65.

RING - C :

Considerable variations are generally found for the chemical shifts of the C-ring protons among the several flavanoid classes. For example, the C-3 proton in flavones gives a sharp singlet near τ 3.7. The C-2 proton of isoflavones is normally observed at about τ 2.3, while the C-2 proton in flavanones is split by C-3 protons into a doublet of doublet (J_{cis} = 5Hz, J_{trans} = 11Hz) and occurs near τ 4.8. The two C-3 protons occur as two quartets ($J_{H_{3a-3b}}$ = 17Hz) near τ 2.3. However, they often appear as two doublets since two signals of each quartet are of low intensity. The C-2 proton in dihydroflavanols appears near τ 5.1 as a doublet (J = 11Hz) coupled to the C-3 proton which comes at about τ 5.8[82] as doublets.

In the structure elucidation of biflavonoids, certain useful information can be obtained by comparison of their NMR spectra with those of their corresponding monomers. Such a choice, however, is compelling but by no means infalliable. Comparison of the NMR spectra of methyl and acetyl derivatives of a biflavonoid with those of biflavonoids by the same series as well as those of biflavonoids of the other series in which at least one monoflavonoid unit is similarly constituted is very helpful in assigning each and individual proton and the position of the methoxy groups. The problem of interflavonoid linkages has been successfully solved by solvent induced shift studies of methoxy resonance and lanthinide induced shift studies.

In biphenyl type of biflavones such as amentoflavone, cupressuflavone, agathisflavone etc., the peaks of the ring protons involved in interflavanoid linkage appear at some what lowerfield (0.5 ppm) as compared with the peaks of the same protons in monomer due to extended conjugation.

^{13}C-NMR SPECTROSCOPY OF BIFLAVONOIDS :

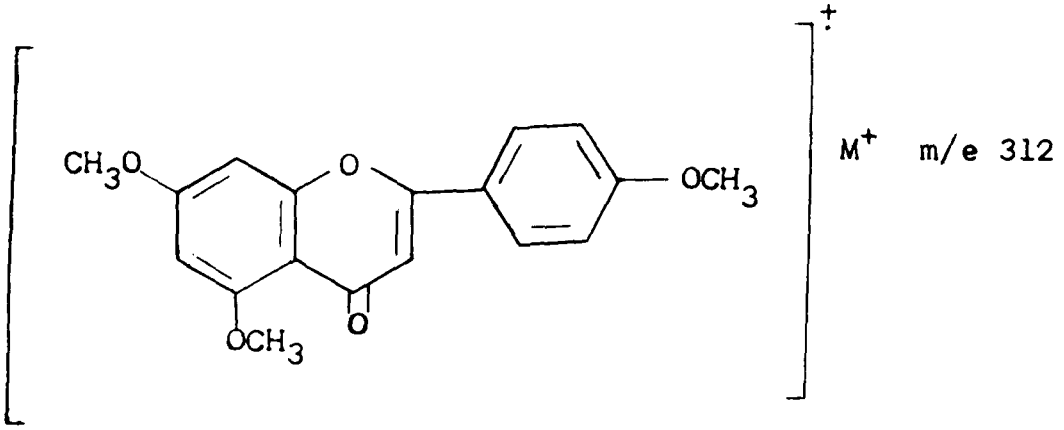
^{1}H-NMR spectroscopy involving shifts of the methoxy signals in the spectrum of permethyl ether, upon progressive addition of deuter-obenzene[106] has been used for the

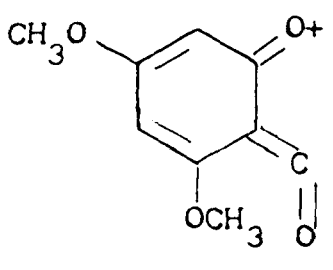
determination[39,53] of interflavonoid linkage. The shift of the signal occurs if one position ortho- to a given methoxy group is unsubstituted. Though applied successfully in many cases, this method is restricted in its applicability. Thus in case of hepta-O-methyl Saharanflavones[20] one methoxy signal does not shift as at all, on addition of C_6D_6, supporting a (I-3,II-6) linked structure, in spite of the fact that a (I-3,II-8) linkage was later confirmed by synthesis. The use of the paramagnetic shift reagent [Eu $(fod)_3$] helped to differentiate the signals due to H-3, H-6 and H-8, in 5,7-dimethoxyflavonoids[99] and this has been extended to the biflavonoid permethyl ethers[65,107]. However, since both flavonoid moieties are complexed, different shifts may result from the same substituent on each nucleus. Hence a method of wider applicability is necessary for an unambiguous determination of the interflavonoid linkage in such compounds. The assingment of the signals in the ^{13}C-NMR spectra of ten oxygenated biflavanoids was achieved on the basis of off-resonance and protons coupled spectra and by analogy with published values[101,108] for the monomeric compounds. This method obviates the necessity of preparing the permethyl ethers which are obligatory for the ^{1}H-NMR solvent induced shift studies. As a consequence therefore, this method has potential also for the location of methoxy

substitution directly in a naturally occurring methylated biflavonoid.

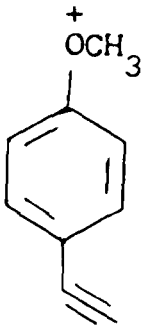
MASS SPECTROMETRY :

The mass spectra of a wide variety of organic natural products have been studied only during the last few years. Recently a number of papers on the evaluation of structure fragmentation pattern relationship in mono and biflavanoyl have appeared [109-112]. The principal mode of fragmentation in flavones and flavanones is retro-Diels-Alder reaction. The apigenin trimethyl ether [113] gives molecular ion m/e 312 as the base peak, further fragmentation by RDA yields ion at m/e 180, m/e 132 and m/e 135.

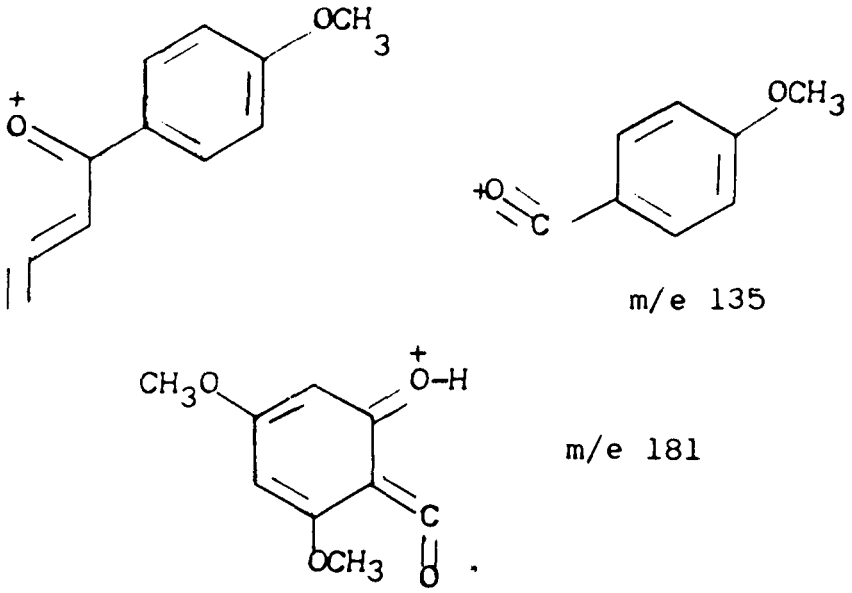




m/e 180



m/e 132



m/e 135

m/e 181

[Hydrogen aquisition by ion at m/e gives ion m/e 181]

Seshadri et al.[113] have reported that the fragmentation pattern of biphenyl type of biflavonoids viz. cupressuflavone hexamethyl ether and amentoflavone hexamethyl ether are similar, molecular ion being the base peak in each case. There are differences in the intensities of the corresponding ions in their spectra, chiefly due to structural variations. Steric factors also seem to play an important role in influencing the break down mode and internal condensations. These factors become so much dominant in agathisflavone hexamethyl ether, that the ion at m/e 311 appears as base instead of molecular ion m/e 622.

The mode fragmentation of hinokiflavone pentamethyl-

ether which contains a biphenyl ether system, is considerably different from those of amentoflavone, cupressuflavone and agathisflavone hexamethyl ethers. The base peak in this case appears at m/e 313 and the molecular ion (m/e 608) amounts to 39% of this peak. This may be attributed to the easily ruptu-rable biphenyl ether linkage[113], hydrogen transfer then leads to the m/e 313 fragment. The fission of the ether bridge can take place in two ways :

I. Routine-1 giving the ions at m/e 297 and m/e 311 and

II. Route-2 yielding the ions at m/e 281 and m/e 347.

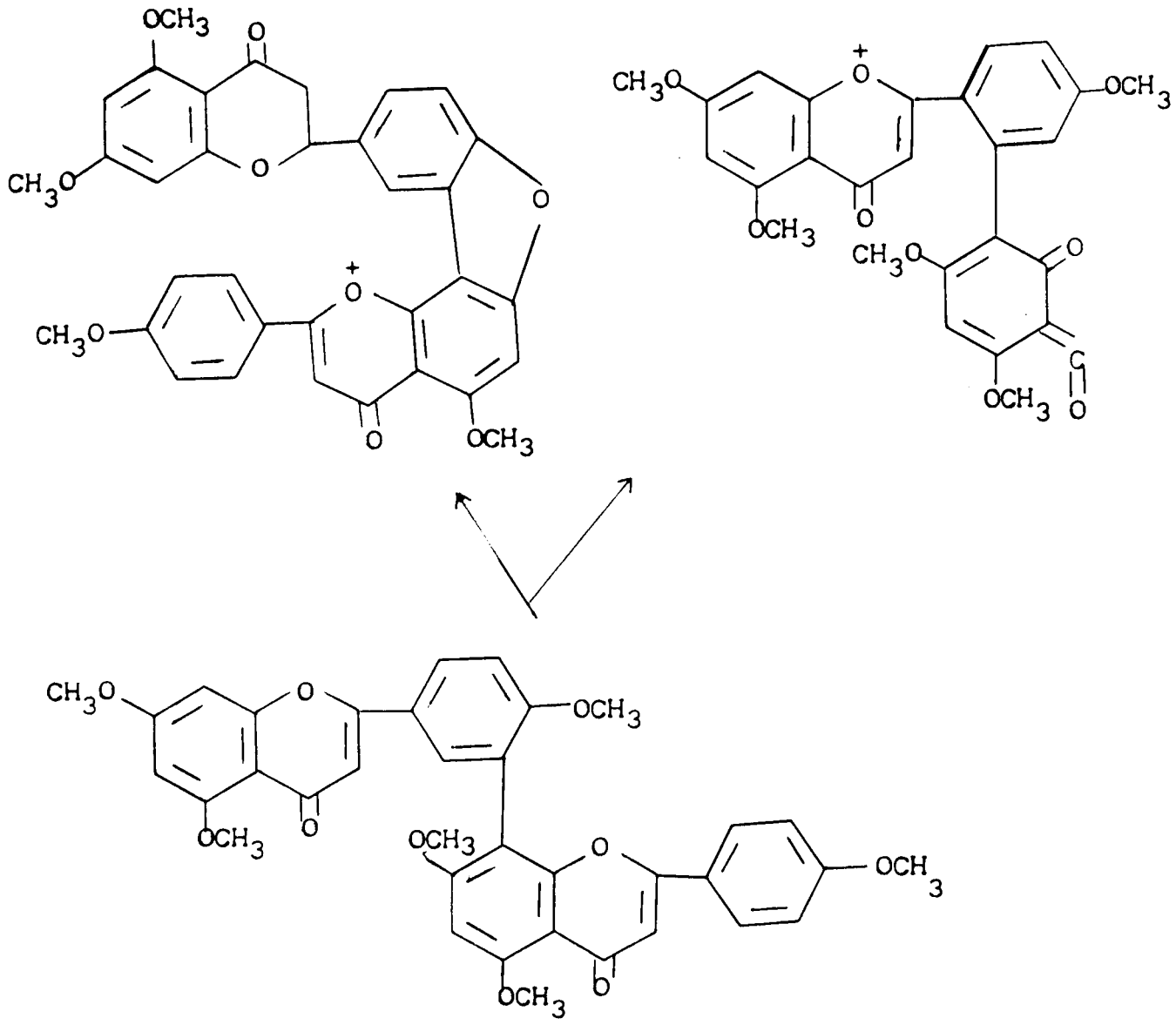
However, the observation that the 313 ion is most intense suggests that route-1 is favoured i.e. the bond between the oxygen bridge and the highly oxygenated phenyl ring breaks preferably[112].

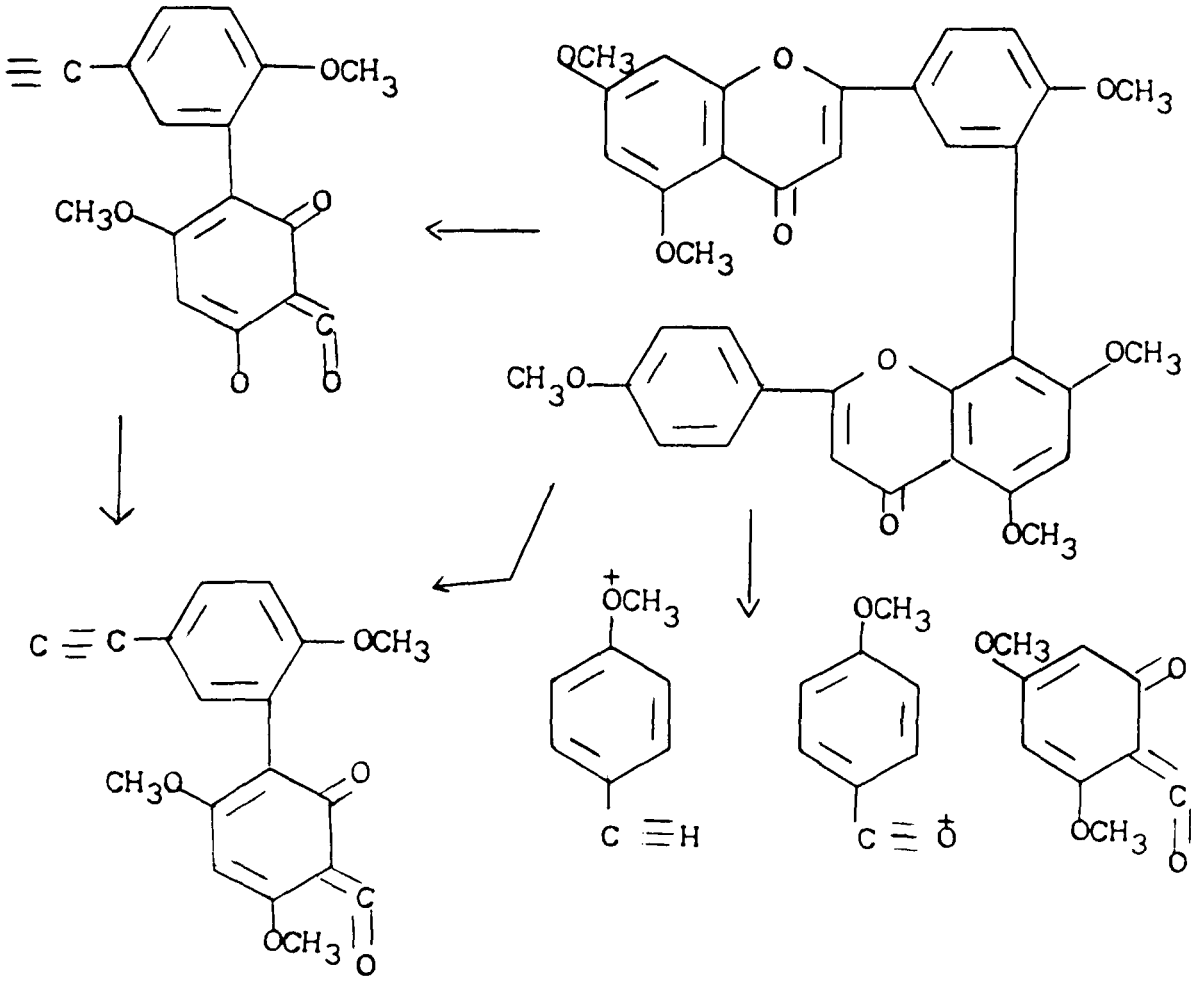
Amentoflavone hexamethyl ether (I) :

The mode of fragmentation is shown in chart.

Main Peaks :

622 (100), 621 (33), 592 (8), 576 (10), 321 (2), 311 (5), 245 (5), 181 (2), 180 (3), 135 (16) and 132 (3).





SYNTHESIS OF BIFLAVONOIDS :

The synthetic approaches to members of biflavone families falls into five categories. The three distinct categories are as follows :

(a) Coupling of two flavone nuclei by Ullman reaction[114-115].

(b) Ullman synthesis of suitably substituted biphenyls and biphenyl ethers followed by their heteroannulation to flavones[116-117].

(c) Phenol oxidative coupling[118-120].

DISCUSSION

The genus[121] Biota belonging to the family cupressaceae, comprising of more than 10 species, evergreen shurbs are native to China, Japan, Formosa, North America and Kashmir. Some of the species are now cultivated in most Asian countries. This genus is mostly used for perfumes as it contains perfume ingradients. Most important and vital use of this genus is that its plant materials are used as disinfectants, insecticides and as house-hold cleaner[122].

Most of the species from this genus contain biflavonoids having following linkages[23,25,123-125] viz. (I-3',II-8) amentoflavone, (I-4'-O-II-6) hinokiflavone, (I-8,II-8) cupressuflavone.

In present work, Biota semipervirens Lin. on phytochemical investigation showed the presence of three types of biflavonoids, amentoflavone, cupressuflavone and robustaflavone.

Extraction of biflavonoidic constituents from the leaves of Biota semipervirens Lin. (cupressaceae) :

Dried and powdered leaves 1 kg were exhaused with petroleum ether (40-60°). The petrol exhausted leaves were refluxed with acetone till the extract was almost colourless. The combined acetone extracts were concentrated at first by atmospheric pressure and then under reduced pressure. A solid was obtained and refluxed with petroleum ether (40-60°), benzene and chloroform successively till the solvent in each case was almost colourless. The residue was treated with boiling water and insoluble left behind was refluxed with EtOAc. The ethylacetate portion was concentrated and it responded colour test for flavonoids. Purification of biflavonoids by column chromatography on elution with EtOAc and Acetone (1:1) gave a biflavonoid mixture. This mixture was concentrated to yield a solid mass which was subjected to thin layer chromatography. On TLC, three components BSI, BSII and BSIII were separated.

Extensive chromatography of the EtOAc extract of the present plant material has afforded above biflavonoidic compounds [BSI to BSIII].

Structure elucidation of these compounds by chemical and spectroscopic methods are discussed as :

Structure of BSI :

The compound BSI, m.p. 335°, M^+ at m/e 538 analysed for $C_{30}H_{18}O_{10}$. It gives red colouration with Mg-HCl and light green with alcoholic $FeCl_3$, indicating flavonoidphenolic hydroxyls. Its U.V. spectrum recorded in MeOH shows absorption maxima at λ_{max} 218, 270, 290 and 330 nm. The typical bathochromic shift in the absorption maxima on addition of NaOH and $AlCl_3$ followed by HCl, indicates the presence of hydroxyls at 5, 7 and 4 positions. The IR(KBr) spectrum exhibits hydroxyl band at ν_{max} 3400 cm^{-1}. The band at ν_{max} 1650 cm^{-1} is due to carbonyl group. Besides this, the spectrum contains other aromatic bands. Further confirmation of the structure is achieved by chemical and spectral analysis of its derivatives.

Methylation of BSI :

BSI on methylation with dimethylsulphate in dry acetone and potassium carbonate, gives hexamethyl ether (BSIM), m.p. 226-27°, $M^{+\cdot}$ at m/e 622 analysed for $C_{36}H_{30}O_{10}$.

The ^1H-NMR data of (BSIM) is tabulated below and is consistant with proposed structure which is fortified by MS fragmentation of methylether of parent compound.

Chemical Shifts of Protons of BSIM :

Assignment	Signal
H-I-8	3.54 (1H, d, J=3Hz)
H-I-6	3.68 (1H, d, J=8Hz)
H-II-6	3.37 (1H, s)
H-I-3	3.50 (1H, s)
H-II-3	3.42 (1H, s)
H-I-6'	2.06 (1H, q, J_1=9Hz, J_2=3Hz)
H-I-2'	2.15 (1H, d, J=3Hz)
H-I-5'	2.90 (1H, d, J=9Hz)
H-II,2',6'	2.62 (2H, d, J=9Hz)
H-II,3',5'	3.27 (2H, d, J=9Hz)
OMe-II-5'	5.94 (s, 3H)
I-5,I-7,II-7	6.04, 6.14, 6.18
I-4',II-4'	6.24, 6.28 (s, 3H each)

s = singlet, d = doublet, q = quartet,
spectra run in $CDCl_3$ at 100 MHz.
[TMS as internal standard 10.00].

The ^{1}H-NMR spectrum of BSIM (Fig. I) showed ABX and A_2B_2 systems associated with ring I-B and II-B substituted at positions I-3',4' and II-4' respectively. Thus rings I-B and II-A of the biflavone seemed to be involved in

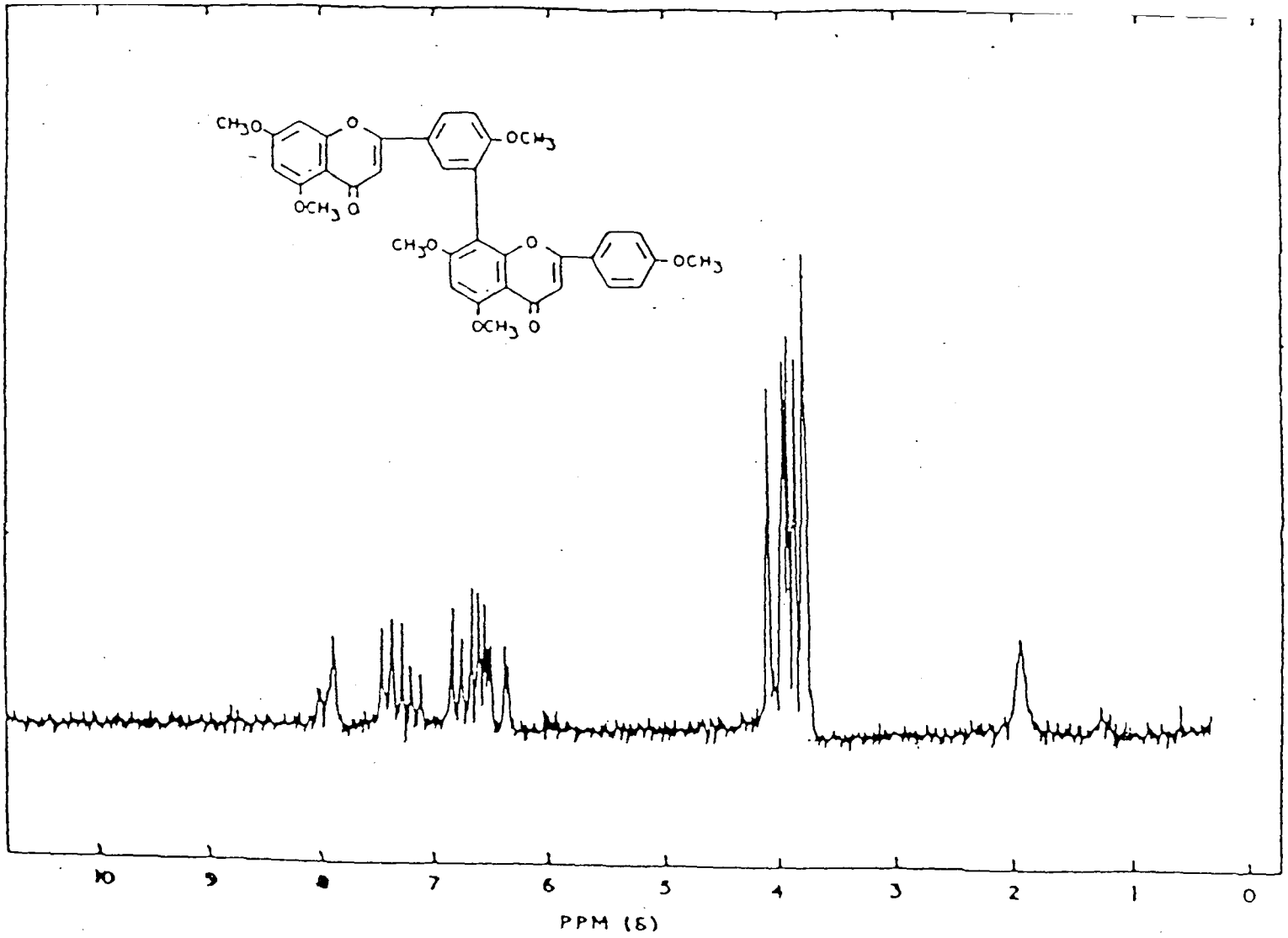


Fig. I

interflavonoid linkage. In particular the values showed that I-5 is linked to either II-6 or II-8. The observation that in biflavones, having aromatic substituents at I-8, I-5-OMe group generally appeared below τ 6.0 led to believe that substituents (flavone unit) in BSIM was located at II-8 and not at II-6. Further all methoxy groups on change of solvent from deutero chloroform to benzene moved upfield as in cupressuflavone hexamethyl ether, showing that every methoxy group had at least one ortho proton, therefore a II-8, rather that II-6 linkage was established.

Acetylation of BSI :

Acetylation of BSI with acetic anhydride and pyridine afforded hexaacetate [BSIA], m.p. 241-242°, M^+ at m/e 790, analysed for $C_{42}H_{30}O_{16}$.

The ^{1}H-NMR data of its acetate is tabulated below :

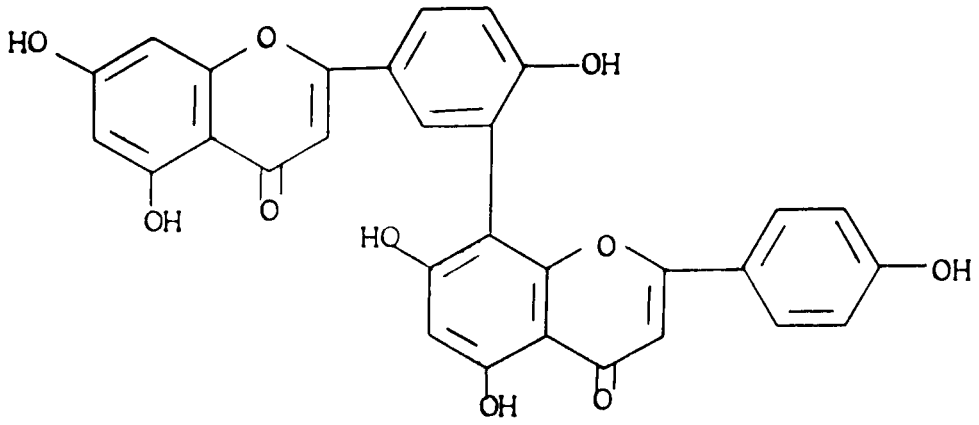
Chemical Shifts of Protons of BSIA :

Assignments	BSIA
H-I-8	2.74 (d, 1H, J=2.5Hz)
H-I-6	3.16 (d, 1H, J=2.5Hz)
H-II-6	2.99 (s, 1H)
H-I-6'	2.02 (q, 1H, J_1=8.5Hz, J_2=2.5Hz)
H-I-2'	1.97 (d, 1H, J=2.5Hz)
H-I-5'	2.54 (d, I-5, J=8.5Hz)
H-II-2',6'	2.51 (d, 2H, J=8.5Hz)
H-II,3',5'	2.94 (d, 2H, J=8.5Hz)
H-I-3,II-3	3.32, 3.35 (s, 1H, each)
OAc-I-5,II-5	7.54, 7.59
OAc-I-7,II-7	7.72, 7.77
OAc-4',II-4'	7.59, 7.95 (s, 3H each)

s = singlet, d = doublet, q = quartet.

Spectrd run in $CDCl_3$ at 100 MHz.

The structure thus assigned for BSI is in full agreement with amentoflavone[126] and its methyl and acetyl derivatives.



I

Structure of BSII :

BSII m.p. 330°C, M^+ at m/e 538, analysed for $C_{30}H_{18}O_{10}$. It gives green colour with alcoholic $FeCl_3$ and orange with Mg-HCl indicative of its flavonoidic-phenolic nature. Its UV spectrum, in EtOH, shows absorption maxima at λ_{max} 230, 269, 334 and 280 nm, which underwent characteristic bathochromic shift on addition of NaOH and $AlCl_3$ characteristic of 5,7-dihydroxyflavonoids.

The I.R.(KBr) of the compound shows bands at ν_{max} 3430 (OH), 1655 (Conj. CO), 1600, 1535, 1470, 1360, 1285, 1240, 1180, 1125, Lo55, 910, 840, 775, 720 cm^{-1}.

To confirm the structure, derivatives of BSII followed by their extensive spectral studies were underwent.

Methylation of BSII :

BSII on methylation with dimethyl sulphate and potassium carbonate in dry acetone, afforded hexamethyl ether BSIIM, m.p. 297-99°, M^+ at m/e 622 corresponding to $C_{36}H_{30}O_{10}$. The methyl ether resonance signals are displayed at δ 4.15 (6H, s, H-5,5'', OMe), 3.85 (6H, s, H-4' and H-4'' OMe) and 3.75 (6H, s, H-7 and H-7''-OMe) in the ^{1}H-NMR spectrum of the compound (Table below).

Chemical Shifts of Protons of BSIIM :

Assignments	BSIIM
H-I-2',6' H-II-2',6'	7.20 (4H, d, J=9Hz)
H-I-3',5' H-II-3',5'	6.75 (4H, d, J=9Hz)
H-I-3, H-II-3	6.58 (2H, s)
H-I-6, H-II-6	6.54 (2H, s)
OMe-I-5,II-5	4.13 (6H, s)
OMe-I-7,II-7	3.75 (6H, s)
OMe-I-4',II-4'	3.84 (6H, s)

s = singlet, d = doublet

Spectrum run in $CDCl_3$ at 100 MHz.

The ^{1}H-NMR spectrum of BSIIM (Fig. II) suggested that the molecule had an axis of symmetry. H-I-3,II-3 and H-I-6,II-6 were distinguished, the former (δ6.58) appearing at the characteristic position for the H-I-3 of a flavone. There was a clear A_2B_2 pattern of protons associated with rings I-B and II-B, indicating the presence of methoxy groups at I-4' and II-4' positions. Two methoxy groups had value below 3.75. These were the I-5 and II-5 methoxy group which were present in two different monoflavonoid unit of the biflavone. The value (below 3.75) is the characteristic

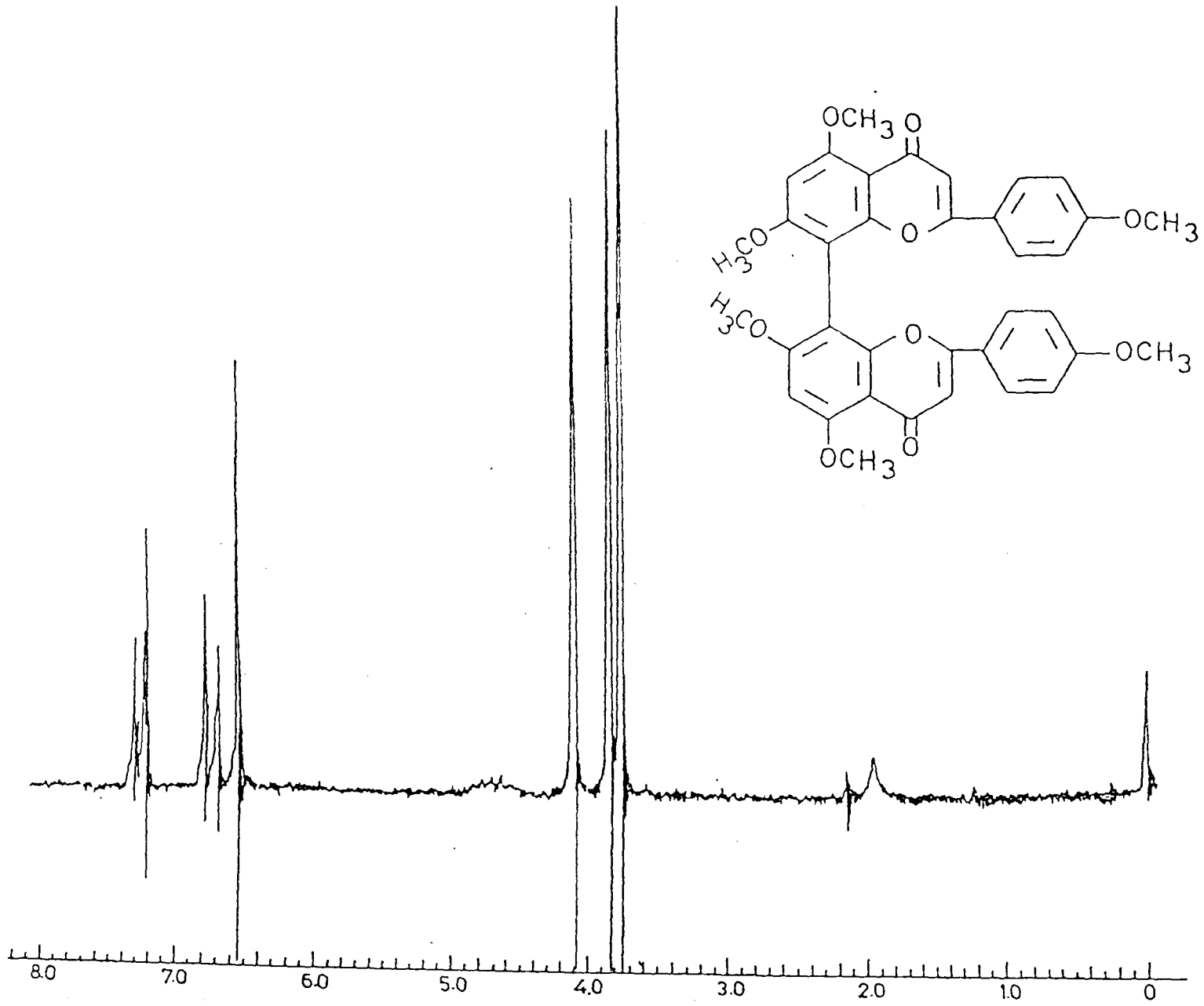


Fig. II

of such groups in I-8, II-8 linked biflavone or in an 8-linked monoflavonoid unit of a biflavone. There was a singlet representing two protons around 6.50. This was assigned to aromatic protons at H-I-6, II-6. The final decission between C-I-8, II-8 and C-I-6, II-6 linkage was taken by benzene induced shift studies of methoxy resonances. The shifts in methoxy resonance as a result of change of solvent from deuterochloroform to benzene show that all methoxy groups shifted upfield as expected for a C-I-8, II-8 linkage.

Acetylation of BSII :

Acetylation of BSII with acetic anhydride and pyridine yielded hexaacetate BSIIA, m.p. 253-56°C, M^+ at m/e 790, analysed for $C_{42}O_{30}O_{10}$. Its ^{1}H-NMR ($CDCl_3$) contains resonance signals at δ 2.50 (s, 6H), 2.26 (s, 6H), 2.10 (s, 6H) according for the acetoxy protons. Table below gives the chemical shifts and multiplicities of the rest of protons.

Chemical Shifts of Protons of BSIIA :

Assignments	BSIIA
H-I-2',6' H-II-2',6'	7.30 (4H, d, J=8.5Hz)
H-I,3',5' H-II,3',5'	7.04 (4H, d, J=8.5Hz)

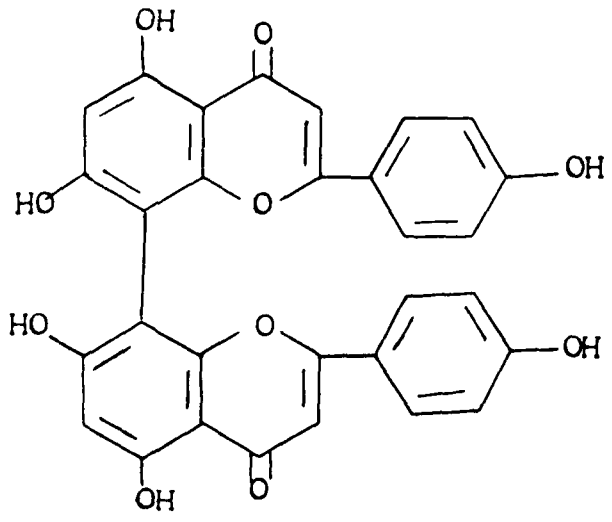
Contd...

H-I-3, H-II-3	6.61 (2H, s)
H-I-6, H-II-6	7.12 (6H, s)
OAc-I-5, II-5	2.50 (6H, s)
OAc-I-7, II-7	2.10 (6H, s)
OAc-I-4',II-4'	2.26 (6H, s)

s = singlet, d = doublet,

Spectrum run in $CDCl_3$ at 100 MHz.

The spectral data of BSII, its acetate and methyl-ether are in full agreement with symmetrically linked biflavonoid, cupressuflavone reported earlier from Cupressus torlusa[46a] and thus assigned structure for cupressuflavone as :



II

Structure of BSIII

BSIIIM, m.p. 305-8°C, obtained by methylation of BSIII, followed by preparative TLC, was found identical in m.p., R_f values and fluorescence in UV light to an authentic sample of robustaflavone hexamethyl ether[23, 127]. The results of PMR studies are given below in Table :

Chemical Shifts of Protons of BSIIIM :

Signals	No. of Protons	J Hz	Assignments
3.66 (d)	1	2.5	H-I-6
3.43 (d)	1	2.5	H-I-8
3.12 (s)	1	-	H-II-8
3.36 (s)	2	-	H-I-3, II-3
2.91 (d)	1	9.0	H-I-5'
2.98 (d)	2	9.0	H-II-3',5'
2.14 (d)	3	9.0	H-II-2',6', I-6'
2.19 (d)	1	2.5	H-I-2'
6.07, 6.39	6	-	OMe-I-5, II-5
6.12, 6.14	6	-	OMe-O-4', II-4'
6.14, 6.18	6	-	OMe-I-7, II-7

s = singlet, d = doublet

Spectrum run in $CDCl_3$ at 100 MHz.

The ^{1}H-NMR spectrum of BSIIIM showed clearly that the molecule was not symmetrical. The appearance of two 1H doublet at γ 3.66 and 3.43 with J values (2.5Hz) characteristic of metacoupled protons and ^{1}H singlet at γ 3.12 suggest that at least one of the two A rings was involved in interflavonoidic linkage. There was evident, a set of A_2B_2 protons at γ.2.98 and 2.14 with J values (9Hz), characteristic of ortho:coupled protons and three other aromatic protons in this region which showed the ABC pattern of B rings. Thus one of B-rings involved in linkage through 3'-position. This linkage could not be through the 3-position as there was evident a 2H singlet at 3.36. The pattern of aromatic signals was more or less like that of amentoflavone hexamethyl ether[17a].

Unlike amentoflavone hexamethyl ether, however no methoxy group appeared below γ 6.00 and one methoxy group appeared at · 6.39, a value much higher than any methoxy group of amentoflavone hexamethyl ether. If the linkage was considered through the 6-position, the signal at γ6.39 could be assigned to 5-OCH_3 at the 6-linked flavone unit in analogy with I-5-OCH_3 of agathisflavone hexamethyl ether.

The mode of interflavonoid linkage as (I-3',II-6) in

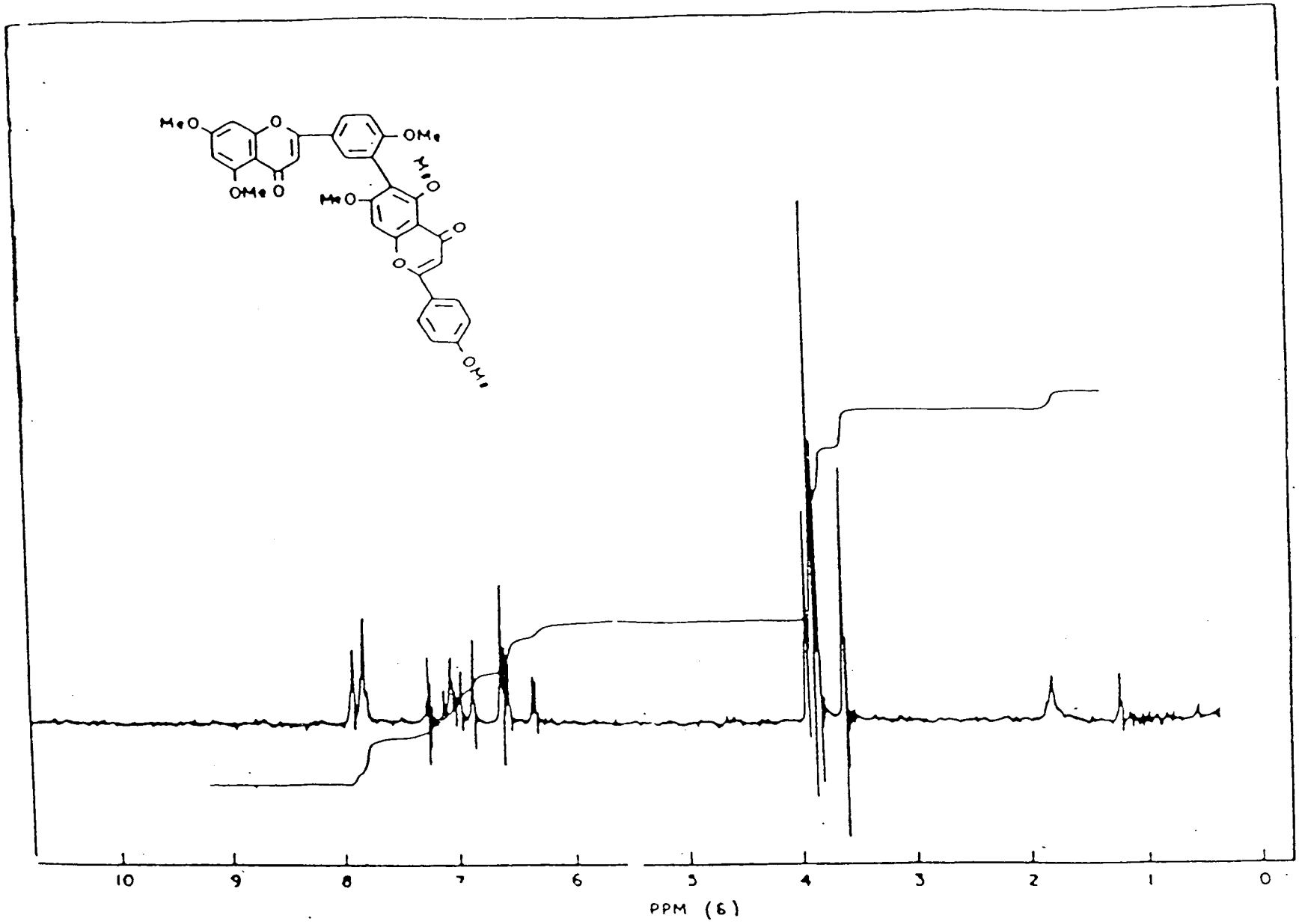
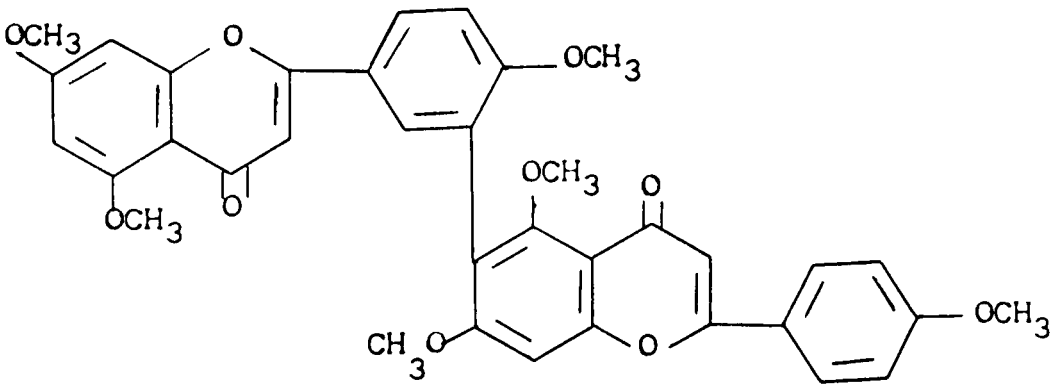


Fig. III

BSIIIM was further established from benzene induced solvent shift studies of methoxy resonances. On change of solvent from $CDCl_3$ to C_6H_6, methoxy group at τ 6.39 move downfield, whereas the other five showed up-field shifts like penta-O-methyl hinokiflavone[71] and hexa-O-methyl agathisflavone[17b]. This findings is compatible with the structure of BSIIIM, because of OCH_3-II-5 in this case is located in similar environments as OCH_3-II-5 of penta-O-methyl hinokiflavone and OCH_3-I-5 of hexa-O-methyl agathisflavone. The above mode of linkage gains further support from mass spectral studies of BSIIIM. The presence of m/e 311 as a major peak in BSIIIM and hexa-O-methyl agathisflavone but very minor one in hexa-O-methyl amentoflavone is of considerable significance. This may be attributed to facile carbon-carbon cleavage in BSIIIM and hexa-O-methyl agathisflavones due to steric reasons.

BSIIIM, therefore has been assigned the structure, I-4',II-4',I-5,II-5,I-7,II-7-hexa-O-methyl (I-3',II-6) biflavone.



III

EXPERIMENTAL

Extraction of biflavonoids from the leaves of *Biota semipervirense* Linn. (Cupressaceae) :

Dried and powdered leaves 1.0 kg were exhausted with petroleum ether (40-60°). The petrol exhausted leaves were refluxed with acetone till the extract was almost colourless. The combined acetone extracts were concentrated first at atmospheric pressure and then under reduced pressure. A gummy dark brownish green mass was obtained. On its purification, a solid green residue was obtained. It was refluxed with petroleum ether (40-60°), benzene and chloroform successively, till the solvent in each case was almost colourless. The residue left behind was then treated with boiling water. The insoluble fraction (gummy mass) was removed, dried and refluxed with ethyl acetate for several hours. The ethyl acetate extract was evaporated to give a dark brown solid, which responded to usual colour tests for flavonoids.

Purification of biflavonoid mixture - Column chromatography :

A well stirred suspension of silica gel (150 gm) in dry

petroleum ether (40-60°) was poured into a column (150 cm long and 50 mm diameter). When the adsorbent was settled, the excess of petroleum ether was allowed to pass through column. The dark brown solid (10 gm) adsorbed in silica gel was added to the column. The column was successively eluted with petroleum ether, benzene, chloroform, ethyl acetate - benzene (1:1, 1:2), ethyl acetate and acetone. The last four fractions gave usual flavonoid colour tests which were combined and solvent distilled off to give dark brown solid mass (7 gm).

Separation of biflavonoid mixture - Preparative layer chromatography :

Using a thin layer spreader (Desaga, Heidelberg), glass plates (40 x 20 cms) were coated with a well stirred suspension of silica gel (50 gm in 95 ml of water) to give a layer approximately 0.5 mm in thickness. After drying for 2 hours at room temperature, the plates were activated at 110-20° for an hour in an oven.

The complexity of the biflavonoid mixture, obtained after purification by column chromatography was examined by TLC using the following solvent system :

(a) Benzene - Pyridine - Formic acid [B:P:F (36:9:5)]

(b) Toluene - Ethyl formate - Formic acid [T:EF:F(5:4:1)]

In solvent system (a), the biflavonoid mixture showed three compact brown spots in UV light. They were labelled as BSI, BSII and BSIII (R_f 0.17, R_f 0.37 and R_f 0.54) respectively. There was a marked difference in R_f values in B:P:F system so it was run for quantitative separation.

The brown solid (7 gm) obtained from column was dissolved in pyridine and the solution so obtained was applied to plates with the help of mechanical applicator (Desaga, Heidelberg), 2 cm from the lower edge of the plates. The plates mounted on a stainless steel frame placed in a Desaga glass chamber (45 x 22 x 22 cm) containing 500 ml of developing solvent (BPF, 36:9:5). When the solvent front had travelled 18 cm from the starting line, the plates were taken out and dried at room temperature. The position of the bands were marked in UV light. The marked pigment zones were scraped with the help of spatula and eluted with dry acetone. The elute in each case was distilled off to give oily liquid which on addition of water yielded yellow precipitate. It was filtered, washed with water and dried. Homogenity of the pigments was again checked by TLC using different solvent systems. The fraction thus obtained gave pure BSI, BSII and BSIII components. The complexities of all the fractions were studied by TLC examination of their fully methylated products.

BSI Methylation :

BSI (100 mg), anhydrous potassium carbonate (2 gm), dimethylsulphate (1 ml) and dry acetone (400 ml) was refluxed on water bath for 12 hours. A small portion of the reaction mixture was taken out in a test tube and tested for alc. $FeCl_3$ reaction. Refluxing continued untill it gave a negative alc. $FeCl_3$ test. It was then filtered and the residue was washed several times with hot acetone. The filterate and washing was combined and evaporated to dryness. The yellow oily mass left behind was treated with petroleum ether and then dissolved in chloroform. The chloroform solution was washed with water, dried over anhydrous Na_2SO_4 and concentrated to give a crude solid. It was purified on a silica gel column using chloroform as eluent.

The methylated product on TLC examination was found to be amentoflavone hexamethyl ether (R_f values, characteristic fluorescene in UV light).

I-4',II-4'-1,5,II-5,I-7,II-7-Hexa-O-methyl(I-3,II-8) biflavone BSIM :

It was crystallized from $CHCl_3$ - MeOH as colourless needles, m.p. 226-27°.

^{1}H-NMR ($CDCl_3$) - Values on τ Scale :

3.54 (1H, d, J=3Hz, H-I-8), 3.68 (1H, d, J=3Hz, H-I-6), 3.37 (1H, s, H-II-6), 3.50 (1H, s, H-I-3), 3.42(1H, s, H-II-3), 2.06 (1H, q, J_1=9Hz, J_2=3Hz), 2.15 (1H, d, J=3Hz, H-I-2'), 2.90 (1H, d, J=9Hz, H-I-5'), 2.62 (2H, d, J=9Hz, H-II-2',6'), 3.27 (2H, d, J=9Hz, H-II-3',5'), 5.94 (3H, s, OMe-II-5), 6.04, 6.14, 6.24, 6.28 (3H, each s, OMe-I-5, II-5, I-7, II-7, I-4', II-4').

Acetylation of BSI :

Anhydrous BSI (100 mg) was heated with pyridine (1.5 ml) and acetic anhydride (3 ml) on a water bath for 3 hours. It was then cooled to room temperature and poured onto crushed ice. The separated solid was filtered, washed with water and dried.

I-4',II-4',I-5,II-5,I-7,II-7-Hexaacetoxy[I-3',II-8] biflavone (BSIA) :

It was crystallized from $CHCl_3$-MeOH as colourless needles, m.p. 240-42°.

2.74 (1H, d, J=2.5Hz, H-I-8), 3.16 (1H, d, J=2.5Hz, H-I-6), 2.99 (1H, s, H-II-6), 2.02 (1H, q, J_1=2.5Hz, J_2=8.5Hz, H-I-6'), 1.97 (1H, d, J=2.5Hz, H-I-2'), 2.54 (1H, d,

J=8.5Hz, H-I-5'), 2.51 (2H, d, J=8.5Hz, H-II-2',6'), 2.94 (2H, d, J=8.5Hz, H-II-3',5'), 3.32, 3.35 (s, 1H each, H-I-3, H-II-3), 7.54, 7.95 (3H each, s, I-5, II-5), 7.72, 7.77 (3H each, s, I-7, II-7), 7.95, 7.99 (3H each, s, I-4, II-4').

Methylation of BSII :

BSII (100 mg) was methylated using dimethyl sulphate and anhydrous potassium carbonate in dry acetone as described earlier. TLC examination of methylated product revealed the presence of a biflavonoid hexamethyl ether (cupressuflavone hexamethyl ether) (R_f, characteristic fluorescence in UV light), m.p. 297-299°C.

I-4',II-4',I-5,II-5,I-7,II-7-Hexa-O-methyl[I-8,II-8] biflavone (BSIIM) :

The product after usual work up was crystallized from $CHCl_3$-MeOH as colourless needles, m.p. 270-279°C.

NMR ($CDCl_3$) Values on δ-Scale :

7.20 (4H, d, J=9Hz, H-I-2',6', II-2',6'), 6.75 (4H, d, J=9Hz, H-I-3',5', II-3',5'), 6.58 (2H, s, H-I-3, II-3), 6.54 (2H, s, H-I-6, II-6), 4.13 (6H, s, OMe-I-5, II-5),

3.75 (6H, s, OMe-I-7, II-7), 3.84 (6H, s, OMe-I-4', II-4').

Acetylation of BSII

[(I-4',II-4',I-5,II-5,I-7,II-7-Hexaacetoxy(I-8,II-8)] biflavone (BSIIA) :

BSII was acetylated with pyridine and acetic anhydride as described earlier and crystallized from $CHCl_3$-MeOH as colourless prisms, m.p. 252-53°C.

NMR ($CDCl_3$; Values on δ-Scale) :

7.12 (2H, s, H-I-6, II-6), 7.30 (4H, d, J=8.5Hz, H-I-2',6', II-2',6'), 7.04 (4H, d, J=8.5Hz, H-I-3',5', H-II-3',5'), 6.61 (2H, s, H-I-3, II-3), 2.50 (6H, OAc-I-5, II-5), 2.26 (6H, OAc-I-4', II-4'), 2.10 (6H, OAc-I-7, II-7).

Methylation of BSIII :

BSIII on methylation with dimethyl sulphate and anhydrous potassium carbonate in dry acetone as described earlier, gave a methylated product which on TLC examination revealed the product as robustaflavone hexamethyl ether.

I-4',II-4',I-5,II-5,I-7,II-7-Hexa-O-methyl(I-3',II-6) biflavone (BSIIIM) :

The fraction BSIIIM on crystallization ($CHCl_3$-MeOH) gave colourless needles, m.p. 305-308°C.

^{1}H-NMR ($CDCl_3$) Values on τ-Scale :

3.66 (1H, d, J=2.5Hz, H-I-6), 3.43 (1H, d, J=2.5Hz, H-I-6), 3.43 (1H, d, J=2.5Hz, H-I-8), 3.12 (1H, s, H-II-8), 3.36 (2H, s, H-II-8), 3.34 (2H, s, H-I-3, II-3), 2.91 (1H, d, J=9Hz, H-I-5'), 2.98 (2H, d, J=9Hz, H-II-3',5'), 2.14 (3H, d, J=9Hz, H-I-2',6', I-6'), 2.19 (1H, d, J=2.5Hz, H-I-2'), 6.07, 6.39 (6H, OMe-I-5, II-5), 6.12, 6.14 (6H, OMe-I-4', II-4'), 6.14, 6.18 (6H, OMe-I-7, II-7).

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